Isolation and analysis of a symbiosis-regulated and Ras-interacting vesicular assembly protein gene from the ectomycorrhizal fungus *Laccaria bicolor*

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Summary

• A yeast two-hybrid library prepared from *Laccaria bicolor* × *Pinus resinosa* mycorrhizas was screened using a *LbRAS* clone, previously characterized, as a bait to isolate LbRAS interacting signaling-related genes from *L. bicolor*.

• Using this method, a novel line of Ras-interacting yeast two-hybrid mycorrhizal (*Rythm*) clones were isolated and analysed for their symbiosis-regulation. One such clone identified (*RythmA*) had homology to Ap180-like vesicular proteins.

• Sequence homology and parsimony-based phylogenetic analysis showed its relatedness to Ap180-like proteins from other systems. DNA analysis suggested that *L. bicolor* had one or two copies of the *RythmA* gene.

• An RNA analysis showed that the expression of *RythmA* could be detected 36 h after interaction with the host, which follows the expression of *Lbras*. Immunolocalization of LbRAS near dolipore septum of the fungal cells in the Hartig net area suggests that RythmA protein may be involved in the transport of signaling proteins such as LbRAS.

Key words: yeast two-hybrid system, ectomycorrhizas, LbRAS-interacting Ap-180 like proteins, gene expression, *Laccaria bicolor*.

Introduction

Ectomycorrhizas are mutualistic symbiotic associations between plant roots and compatible fungi. They are complex organs consisting of plant root and fungal mycelial tissues. Ectomycorrhizae play a key role in maintaining and enhancing the health of a broad range of plants (Harley & Smith, 1983; Smith & Read, 1997). The fungal partner penetrates the plant root epidermis and grows intercellularly between cortical cells. A network of fungal hyphal tissue growing in and around the plant roots facilitate the exchange of nutrients (Harley & Smith, 1983; Smith & Read, 1997). The fungal partner depends on its host for carbon and energy source while helping the plant host by facilitating the absorption of water and essential nutrients such as phosphorus, and nitrogen present in low abundance in the soil (Harley & Smith, 1983; Smith *et al.*, 1994; Hampp *et al.*, 1995; Smith & Read, 1997; Nehls *et al.*, 1998, 1999, 2001a; Hibbett *et al.*, 2000; Jargeat *et al.*, 2000; Heinonsalo *et al.*, 2001; Lilleskov & Burns, 2001). Ectomycorrhizal association also leads to increased resistance in plants against disease and pests and provides the ability to withstand unfavorable soil conditions in general (Allen, 1992; Harley & Smith, 1983; Duchesne *et al.*, 1989; Boyle & Hellenbrand, 1990; Farquhar & Peterson, 1991; Galli *et al.*, 1994; Smith & Read, 1997; Shaul *et al.*, 1999; Martin, 2001). Increased nutrient uptake generally results in improved plant health and vigor, leading to an improved ecosystem (Boyle & Hellenbrand, 1990; Gavito *et al.*, 2000; Sharples *et al.*, 2000).

Ectomycorrhizal formation and functioning is characterized by a variety of dynamic molecular events, including extensive signaling and nutrient traffic between the partners eventually leading to sustained nutrient exchange. The signaling interactions between the partners lead to recognition of each other and the establishment of a functional symbiotic organ (Harley & Smith, 1983; Gianinazzi-Pearson & Gianinazzi, 1989; Martin *et al.*, 1994; Martin, 2001). There have been reports of considerable signaling and gene response events between the partners during ectomycorrhizal symbiosis

(Barker *et al.*, 1998; Kim *et al.*, 1998; Martin & Tagu, 1999; Barker & Tagu, 2000; Tagu *et al.*, 2000; Martin, 2001). Because the very purpose and essence of the association is a coordinated nutrient exchange, it is only logical to expect genes related to nutrient traffic to be regulated during the symbiotic phenomenon. Precise control of such nutrient exchange events requires extensive signaling between the partners and hence a coupling of regulation of signaling as well as nutrient traffic is likely. This would require upregulation and/or downregulation of several genes in both the partners and there have been reports of regulation of several genes, including genes involved in signaling, biosynthesis, metabolism, morphogenesis, cytoskeletal reorganization and vesicular traffic in both the partners during the symbiosis. (Balasubramanian *et al.*, 2002; Hilbert *et al.*, 1991; Martin *et al.*, 1994; Tagu *et al.*, 1996; Kim *et al.*, 1998, 1999a,b; Martin, 2001; Nehls *et al.*, 2001a,b; Sundaram *et al.*, 2001; Podila *et al.*, 2002; Peter *et al.*, 2003).

We have identified a gene (*RythmA*) from the ectomycorrhizal fungus *Laccaria bicolor* (an ectomycorrhizal fungus with wide host range) that appears to code for AP180-like vesicular protein reported in other systems. Ap180 gene products were first reported to be found in clathrin-coated vesicles and have been implicated in vesicular assembly and cargo sorting. Interactions with other vesicular proteins and GTPases have been reported in higher eukaryotic systems as well as *Saccharomyces cerevisiae* (Brodsky, 1988; De Camilli *et al.*, 1996; Schekman & Orci, 1996; Tang *et al.*, 1997; Wendland & Emr, 1998; Hao *et al.*, 1999; Marsh & McMahon, 1999; Greener *et al.*, 2000). Because there have been previous reports of vesicular traffic protein regulation during ectomycorrhizal interactions and vesicular turnover in ectomycorrhiza (Cole *et al.*, 1998; Kim *et al.*, 1999b), it is reasonable to expect genes coding for vesicular coat and assembly proteins to be involved in the process. However, roles of vesicular proteins in mycorrhizal symbiosis are yet to be fully understood and the regulation of vesicle assembly proteins in the phenomenon has not been previously reported. Here, we report regulation of a fungal *AP180*-like gene in *L. bicolor* in response to symbiotic interactions with its host red pine (*Pinus resinosa*). Further, this fungal gene is part of a novel line of mycorrhizal clones (Rhythm–Ras interacting yeast two-hybrid mycorrhizal clones) that exhibit yeast two–hybrid interactions with a previously characterized symbiosis-regulated *L. bicolor* Ras (Sundaram *et al.*, 2001).

Materials and methods

Media, cultures, and *L. bicolor–P. resinosa* interactions

The *L. bicolor* (Maire) Orton DR170 strain used for our studies was a basidiocarp isolate associated with the roots of red pine obtained in Michigan's Upper Peninsula (provided by D. Richter, School of Forestry, Michigan Technological University, Houghton, MI, USA). The fungus was grown and maintained on MMN medium (Marx, 1969; Bills *et al.*, 1995). *In vitro* interactions and harvesting of interacted fungal tissue have been described previously (Kim *et al.*, 1998, 1999b; Wong *et al.*, 1990). Synthesis of mycorrhizal tissue in soil has been described previously (Bills *et al.*, 1999).

Isolation of cDNA clones coding for Lbras-interacting proteins

Isolation of total RNA from 4 month-old *L. bicolor–P. resinosa* ectomycorrhizal tissues, synthesized under sterile conditions, was carried out as described previously (Kim *et al.*, 1998). Smart cDNA synthesis kit (Clontech, Palo Alto, CA, USA) was used to synthesize cDNA pool ligated with *Sfi* arms as per the manufacturer's instructions. LexA Match Maker Library kit (Clontech) was used to perform the yeast two-hybrid screens (Fields & Song, 1989; Chein *et al.*, 1991; Bartel *et al.*, 1993; Fields, 1993; Bendixen *et al.*, 1994; Fields & Strenglanz, 1994). The mycorrhizal cDNA pool synthesized with the smart cDNA kit was cloned into the Match Maker library vector using a oligonucleotide linkers containing restriction sites for the enzymes *Eco*RI, *Sfi*I and *Xho*I (5′-TCG AGT GGG CCG AGG CGG CCG GAT CCG GGC CAT AAT GGC CG-3′, 5′-TC GAG TGG GCC GAG GCG GCC GGA TCC GGG CCA TAA TGG CCG-3′). Full-length Lbras cDNA was cloned into the Matchmaker LexA-binding domain-containing vector and two-hybrid interactions were performed as per the manufacturer's instructions (Clontech). A previously well-characterized *Ras* clone (*Lbras*) from the ectomycorrhizal fungus *L. bicolor* (Sundaram *et al.*, 2001) was used as bait in a yeast two-hybrid interaction system. About 10 000 colonies were screened for positive interactions by selecting for Leu+ followed by a standard blue/white selection for expression of β-galactosidase (Fig. 1). The plasmids containing the interacting clones were rescued from the yeast as per the manufacturer's instructions (Clontech). *Escherichia coli* DH5α cells were transformed with the rescued plasmids. Plasmid preps from the *E. coli* cells were used for further analysis.

Sequence and phylogenetic analyses

Nucleotide sequencing was carried out with the Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA, USA) and the ABI PRISM 310 genetic analyser (PE Applied Biosystems). For sequence analyses, Sequencing software, version 3.0 (PE Applied Bio-systems), and MacDNAsis software, version 5.0 (Hitachi Instruments, San Jose, CA, USA), were used. Multiple alignment of the sequences was done with MULTALIN (www.toulouse.inra.fr/multalin). A parsimony-based phylogenetic analysis was performed on all the proteins that showed homology to *Rythm* clones using the analysis software PAUP version 4.0b.8 (Sinauer Associates, Sunderland MA, USA) (Farris, 1983; Berlocher & Swofford, 1997).

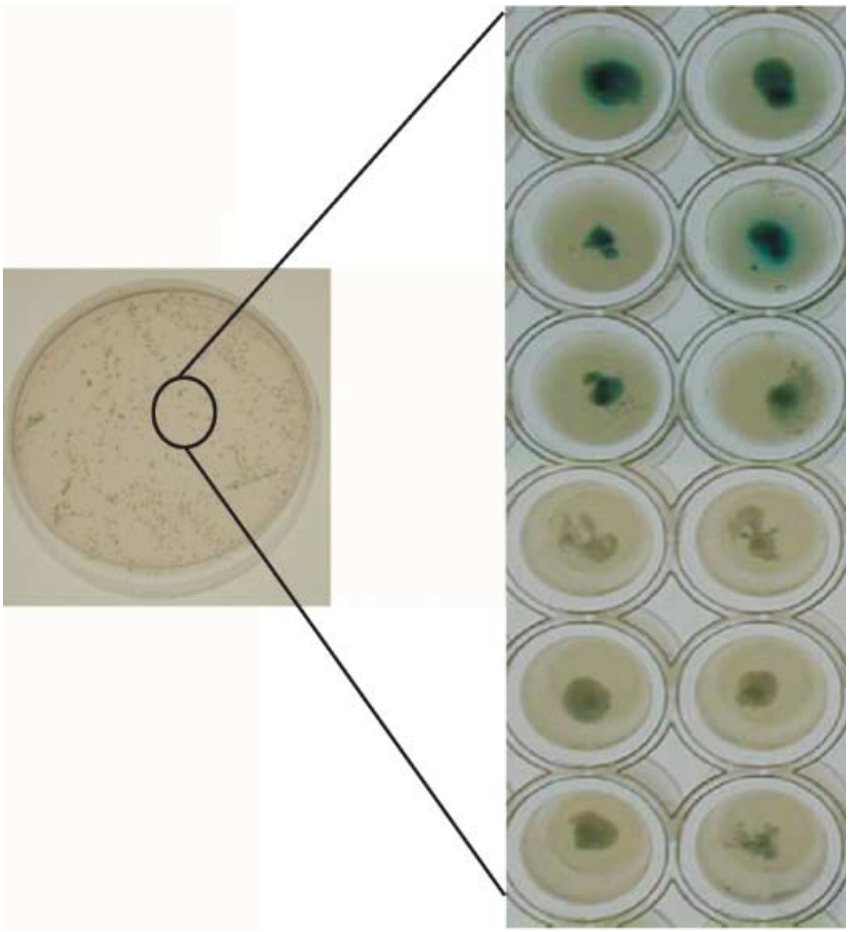

Fig. 1 *Saccharomyces cerevisiae* colonies showing two-hybrid interactions on selection medium containing galactose, raffinose and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) for induction and detection of reporter gene expression. Blue colonies indicate positive interactions between Lbras and Rythm proteins. Colonies in the 96-well plate are duplicates of clones obtained from the initial plate screening for interaction. Spot intensities do not reflect interaction strengths.

Northern and Southern analyses

Total RNA from *L. bicolor*, subjected to interaction with pine seedling roots for 12, 24, 36, 48, 72 and 96 h, respectively, was electrophoresed on agarose gels and transferred to Hybond-N membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA), as described by Kim *et al.* (1998). Total RNA from free-living *L. bicolor* was used as control. A 20 μg sample of RNA was loaded in each lane Gels were stained with SYBR Green (Molecular Probes Inc., Eugene, OR, USA) to determine equal loadings and intensity of RNA. *L. bicolor* genomic DNA isolated from ground mycelia (Reymond, 1987) was digested with restriction enzymes *Bam*HI, *Eco*RI and *Xba*I. A 10-μg sample of genomic DNA was used for each restriction digestion. After resolving the fragments by agarose gel electrophoresis, they were transferred to Hybond-N^+ membranes. The cDNA fragment of pRythmA or pLbras was labeled with ^{32}P-dCTP with the Rediprime DNA labeling kit (Amersham Pharmacia Biotech) and used as a probe in the hybridization analyses of the membrane-bound nucleic acids as described previously (Sambrook *et al.*, 1989; Kim *et al.*, 1999a; Kim *et al.*, 1999b). RNA (50 ng) was used as template for reverse transcription reaction with oligo(dT) primers (Clontech). *RythmA* gene-specific primers (5′-GTG TGC AAG CAG AAG GTA AAC TGG CTG G-3′, 5′-GAC ATA AAC AAC GAA GTT GAG GTA G-3′) were used for the polymerase chain reaction (PCR), and products were resolved electrophoretically on 1% agarose gel and transferred to Hybond-N membranes. Control reverse transcriptase (RT)-PCR was carried out using LbPF6.2 gene-specific primers and probed with *LbPF6.2* cDNA probe (Kim *et al.*, 1998) to compare the levels of expression of *RythmA* in mycorrhizal roots. The RT-PCR experiments were repeated three times to check for consistent results.

Immunolocalization of LbRAS using transmission electron microscopy. Preparation LbRAS recombinant protein was performed as described by Sundaram *et al.* (2001). Briefly, the PCR generated coding region from Lbras cDNA fragment was cloned into expression vector pet22b+ (Novagen, Madison, WI, USA) with a *C*-terminal histidine tag. This construct was used to transform *E. coli*, and expression of the LbRAS recombinant protein, and purification using His-Bind metal chelation resin using protein was carried out as per manufacturer's instructions (Novagen). Purified recombinant protein was used to generate polyclonal antibodies in rabbits (Alpha Diagnostics, San Antonio, TX, USA). The specificity of antibodies was confirmed with purified Lbras recombinant protein using

enzyme-linked immunosorbent assay (ELISA). When Lbras antibodies were preincubated with an excess of recombinant Lbras protein and used for Western blot analysis, it effectively eliminated any signal (Sundaram *et al.*, 2001). Preparation of samples for transmission electron microscopy (TEM) analyses was performed essentially as described by Moore *et al.* (1991). Mycorrhizal roots synthesized under sterile conditions between *P. resinosa* and *L. bicolor* (Bills *et al.*, 1999) were used for TEM analyses. Small (< 4 mm) mycorrhizal and nonmycorrhizal root pieces (same age) were cut, fixed, embedded and sectioned as described (Moore *et al.*, 1991; Sundaram *et al.*, 2001). Cross-sections of roots (100–120 nm) generated with an ultramicrotome equipped with a diamond knife were collected on nickel grids and subjected to immunological analysis. The sections were treated with 1% bovine serum albumin (BSA) and 1 : 30 dilution of rabbit preimmune serum for blocking, then the grids were incubated for 16–20 h with 1 : 1000 dilution of anti-Lbras antiserum in Tris buffered saline (TBS) at room temperature. After washing with TBS, they were treated with TBS containing 1% BSA and 1 : 20 dilution of gold-labeled second antibody (goat antirabbit immunoglobulin Polygold 10 nM; Polysciences, Warrington, PA, USA). After incubation for 1 h, root sections were washed several times with TBS and finally with water and dried. Post-staining with uranyl acetate was performed with some of the samples, as described by Moore *et al.* (1991). The grids were examined with a JEOL JEM-1010 TEM operated at 80 kV. Over 20 sections were observed for immunolocalization patterns of LbRAS. Controls were prepared using the preimmune serum and goat antirabbit immunoglobulin labeled with gold particles.

Results

Isolation of *RythmA* cDNA through yeast two-hybrid screening

A yeast two-hybrid screen was performed with a cDNA library from *L. bicolor–P. resinosa* ectomycorrhizal tissue and a previously characterized *Ras* clone (Lbras) from *L. bicolor* (Sundaram *et al.*, 2001) as bait. This lead to isolation of a novel line of Ras-interacting yeast two-hybrid ectomycorrhizal clones (*Rythm* clones.). Screening of 10 000 yeast colonies resulted in the isolation of over 50 putative clones of which five distinct classes were isolated and analyzed.

Sequence analysis of *RythmA*

One-third of the 50 blue colonies initially selected yielded a plasmid containing the cDNA (*RythmA*) insert that showed sequence homology to other eukaryotic clones coding for AP180-like protein (approx. 50%). The other colonies yielded equal distribution of clones (*RythmB* and *RythmC*) (GenBank accession nos AF420332 and AF420333) and also several partial clones with similarity to HSP16, and rho GTPases (GenBank accession nos BM49310 and BI094653). Sequence analyses using BLAST (www.ncbi.nlm.nih.gov) showed *RythmB* to be a previously uncharacterized gene while *RythmC* showed weak homologies to a receptor kinase (approx. 30%). Because of its abundance in the yeast two-hybrid screen and occurrence of full-length clones, *RythmA* selected for further study. The *RythmA* cDNA was 1325 bp long with a coding region of 798 bp and 5′- and 3′-UTRs of lengths 52 bp and 475 bp, respectively (GenBank accession no. AF420331). The predicted amino acid sequence of RythmA (Fig. 2a) shows the presence of NPF motif, which is characteristic of all known AP180 proteins (De Camilli *et al.*, 1996; Paoluzi *et al.*, 1998; Hao *et al.*, 1999). The Asn–Pro–Phe (NPF) motif has been shown to be involved in protein–protein interactions (Paoluzi *et al.*, 1998). Motif searches with PROSITE (http://motif.genome.ad.jp) profile indicated the presence of five sites for casein kinase II phosphorylation (SLKE, SALD, TGWE, SLYE and SPID) and two phosphorylation sites each for protein kinase C (SLK and TIR) and tyrosine kinase (KLTLDSLY and RCTDGFY). One or more of these sites could serve in a regulatory role controlling RythmA activity in the fungus. A fungal hydrophobin motif was also found in its *C*-terminal end. The

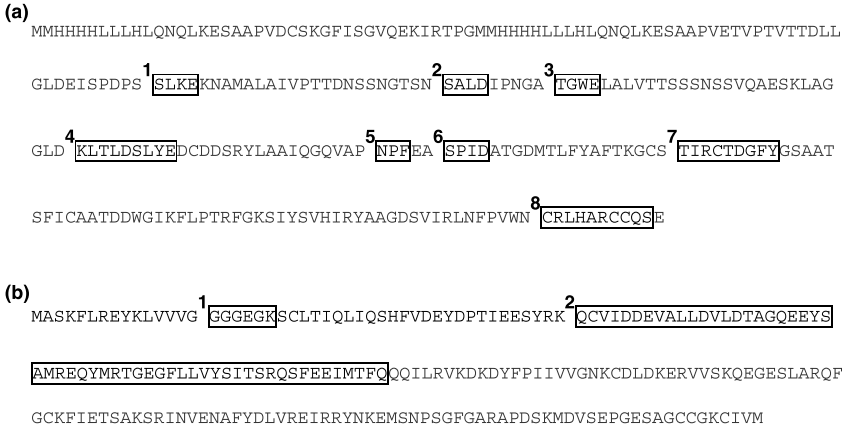

Fig. 2 (a) Amino acid sequence of RythmA showing various domains: 1-5, the casein kinase II phosphorylation sites (SLKE, SALD, TGWE, SLYE, SPID); 1, 6 and 7 include protein kinase C phosphorylation site (SLK, TIR); 4 and 6 also include sites for tyrosine kinase phosphorylation (KLTLDSLY, RCTDGFY); 5, the NPF domain conserved in AP180-like proteins; 8, the fungal hydrophobin region (CRLHARCCQS). (b) Lbras sequence showing an *N*-myristylation site and a conserved ADP ribosylation factor (ARF) family motif (the boxed regions superscripted 1 and 2, respectively).

presence of hydrophobin motifs has been reported previously in symbiosis-regulated genes (Tagu *et al.*, 1996). Also, the size of transcript from northern analysis suggests that the pRythmA insert is a full-length cDNA clone.

Copy number and genomic origins of RythmA

Southern analysis using full length *RythmA* cDNA as a probe showed that the probe hybridized to multiple fragments in each lane containing genomic DNA digested with restriction enzymes (*Bam*HI, *Eco*RI and *Xho*I) indicated on top of the lanes (Fig. 3a). The hybridized DNA fragments were in the size range 1.5–7 kbp. The pLbRythmA-derived probe consisting of the full-length cDNA of *RythmA* was devoid of any sites for the enzymes that were used to digest the genomic DNA. While the lane corresponding to *Bam*HI digestion (Fig. 3a) yielded two bands in the size range 1.5–2 kbp and two more bands in the range 5–7 kbp, the lane corresponding to *Eco*RI digestion yielded three bands in the size range of 3–7 kbp and the lane corresponding to *Xho*I yielded two distinct bands ranging in size between 4 kbp and 5 kbp. The detection of only two fragments in the lane corresponding to *Xho*I digestion indicates that the *RythmA* gene may be present as two copies in the *L. bicolor* genome. However, we were able to detect only one RNA species in Northern blots for *RythmA* (Fig. 3b). Thus, it is possible that *RythmA* may be a single copy gene (see RNA data). The additional bands in lanes corresponding to *Bam*HI and *Eco*RI digestions suggest the presence of additional sites for these enzymes, possibly in introns of the *RythmA* gene in the *L. bicolor* genome. No hybridization was observed when genomic DNA from nonmycorrhizal red pine roots was probed with *RythmA* (Fig. 3a). These data show that *RythmA* is from *L. bicolor*. Although many plant species were known to contain AP180 like sequences, there is no cross reactivity between *RythmA* probe and pine DNA.

Symbiosis–regulation of *RythmA*

In order to determine the actual size of the *RythmA* mRNA and to confirm whether the expression of *RythmA* mRNA was dependent upon ectomycorrhizal symbiotic interactions, Northern blot analysis was performed with full-length *RythmA* probe. The probe hybridized to a single 1.4 kb RNA

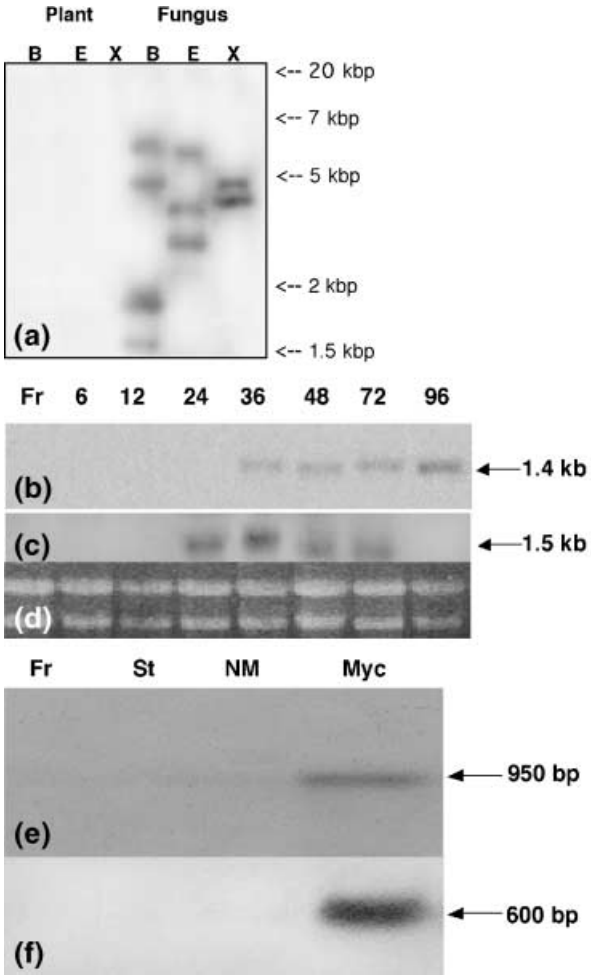

Fig. 3 *RythmA* gene copy number and temporal regulation of *RythmA* expression during *Laccaria bicolor–Pinus resinosa* interactions. (a) Southern analysis of *L. bicolor* (Fungus lanes) and *P. resinosa* (Plant lanes) genomic DNA showing the fungal origin of *RythmA* (fungal lanes). Ten micrograms of genomic DNA was digested with enzymes *Bam*HI (B), *Eco*RI (E) and *Xho*I (X); the fragments were separated by electrophoresis on 1% agarose gels. After transfer to Hybond-N nylon membranes, they were hybridized to the ^{32}P-labeled cDNA probe (insert from the pLbRythmA). (b,c) Northern analysis using RNA samples from *L. bicolor* subjected to interaction with red pine roots for 6 h, 12 h, 24 h, 36 h, 48 h, 72 h and 96 h was carried out. Twenty micrograms of RNA each from the free-living *L. bicolor* (Fr) and *L. bicolor–P. resinosa* interacted tissues were subjected to denaturing electrophoresis on 1% agarose gels. They were transferred to Hybond-N membranes and hybridized to the ^{32}P-labeled *Rhythm A* cDNA probe (insert from the pRythmA) (b) and LbRAS cDNA probe (c). The RNA from *L. bicolor* incubated for 96 h in the absence of pine roots was used as control (Fr). Presence of the *c.* 1.4 kb *RythmA* mRNA was detectable after interaction with the roots for 36 h or more. *LbRAS* expression can be seen from 24 h after interaction, with a peak expression at 36 h. (d) RNA gel showing equal loading of RNA samples. (e,f) Reverse transcriptase polymerase chain reaction analysis of *RythmA* and *LbPF6.2* (control for comparison of relative levels of expression) expression in mycorrhizal roots. Fifty nanograms of RNA from *L. bicolor–P. resinosa* mycorrhizal tissue (lane Myc), free-living *L. bicolor* grown in MMN medium (Lane Fr), *L. bicolor* grown in the interaction medium under carbon starvation conditions (lane St) (Balasubramanian *et al*., 2002) and nonmycorrhizal red pine roots (lane NM) was used as template for reverse transcription. A polymerase chain reaction with *RythmA* and *LbPF6.2* gene-specific primers was performed on the first strands synthesized, and the resultant products were subjected to Southern blot analysis using corresponding ^{32}P-labeled probes as described earlier. Presence of the expected 950 bp fragment for *RythmA* (Panel E) and 600 bp fragment for *LbPF6.2* (Panel F) was detected only in the lane corresponding to *L. bicolor–P. resinosa* ectomycorrhizal tissue.

that was present in samples from the root interacted tissue (Fig. 3b, lanes 36, 48, 72 and 96) at different time-points. The size of this RNA (size markers not shown) was very similar to the size of the insert (1.3 kb) in the pLbRythmA. The small size difference in mRNA could be from 5′-UTR. This RNA could not be detected in the RNA extracted from the free-living fungus (Fig. 3 Panel B, lane Fr), indicating that *RythmA* mRNA in the free-living fungus was absent or below detectable levels. This mRNA was also either absent or below detectable levels in early (earlier than 36 h of interaction) interaction tissues. The RNA samples from *L. bicolor* grown in carbon starvation were also used in this experiment to test if the expression of *RythmA* results from starvation or interaction, as it has been shown in yeast that certain vesicular transport proteins are induced by starvation (Lang *et al.*, 1998). From our results it is clear that starvation did not induce *RythmA*. The same blot when probed with *LbRAS* cDNA probe, showed expression of *LbRAS* beginning from 24 h with a peak expression at 36 h, followed by low levels of expression until 72 h (Fig. 3c). The symbiosis-regulated expression of *RythmA* in mycorrhizal tissues was confirmed by RT-PCR using *RythmA* sequence specific primers followed by hybridization analysis using *RythmA* cDNA probe (Fig. 3e) and no detectable signals were observed from nonmycorrhizal roots. We have used the *LbPF6.2* gene (Kim *et al.*, 1998) as an RT-PCR control to compare the levels of expression of RythmA (Fig. 3f). *LbPF6.2* is also symbiosis regulated and its expression has been linked to interaction between *L. bicolor* and *P. resinosa* (Kim *et al.*, 1998). It is clear that from the expression levels of *LbPF6.2* that the level of expression of *RythmA* is relatively low, similar to *LbRAS* expression (Sundaram *et al.*, 2001).

Phylogenetic analyses of RythmA and Ap180 proteins

Parsimony-based analysis of amino acid sequences of RythmA and other known AP180-like proteins yielded a phylogenetic tree with distinct clusters (Fig. 4). AP180-like proteins from *Caenorhabditis elegans*, *Saccharomyces cerevisiae* and *Arabidopsis thaliana* were grouped away from RythmA and rest of the AP180-like proteins analysed. Further analysis of genome databases of *Fusarium graminearum*, *Aspergillus nidulans*, *A. fumigatus* and *Neurospora crassa*, against *RythmA* sequence resulted in only low complexity matches, which were not significant enough to include in the phylogenetic analyses.

Immunolocalization of LbRAS

In situ localization of LbRAS protein in mycorrhizal tissues using immunoelectron microscopy revealed localized accumulation of LbRAS near dolipore septum (Fig. 5). Ras-like GTPases have been shown to be involved in vesicular transport via interaction with AP180 proteins at the ADP ribosylation factor (ARF) site (Fig. 2b) (De Camilli *et al.*, 1996). It is likely that LbRAS could be transported from cell to cell mediated by RythmA.

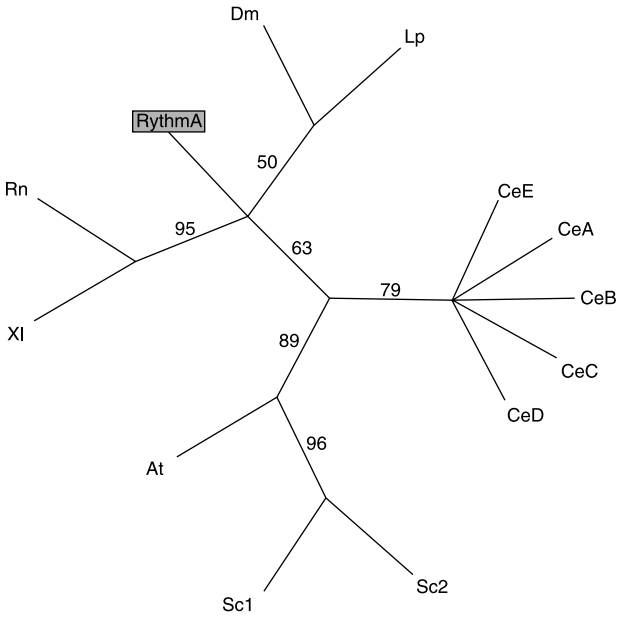

Fig. 4 Phylogenetic analysis of RythmA using PAUP 4.0 software shows its relatedness to other AP180-like proteins. A bootstrap value of 1000 was used to generate an unrooted tree. Sc1, *Saccharomyces cerevisiae* YAP1, X58693; Sc2, *S. cerevisiae* YAP2, X69106; CeA, *Caenorhabditis elegans* AP180-like protein variant A, AF144257; CeB, *C. elegans* Ap180-like protein variant B, AF144258; CeC, *C. elegans* AP180-like protein variant C, Af144259; CeD, *C. elegans* AP180-like protein variant D, AF144260; CeE, *C. elegans* AP180-like protein variant E, AF144261; At, *Arabidopsis thaliana 194* (AP180-like protein), Y10986; Dm, *Drosophila melanogaster* AP180 protein; Xl; *Xenopus laevis* AP180, AF182340; Lp, *Loligo paelai*, AF182339; Rn, *Rattus norvegicus* AP180 protein, X68877.

Discussion

Our key interest was to identify and characterize genes from *L. bicolor* coding for Lbras-interacting proteins as Lbras was shown to be regulated during symbiotic interactions with *P. resinosa* (Sundaram *et al.*, 2001). We employed yeast two-hybrid interactions for this purpose as this technique has been used successfully in many other systems to identify genes coding for interacting proteins (Fields & Song, 1989; Chein *et al.*, 1991; Bartel *et al.*, 1993; Fields, 1993; Bendixen *et al.*, 1994; Fields & Strenglanz, 1994; Hao *et al.*, 1999). Previously, we found that LbRAS expression is regulated both during early stages of interaction and also in the functional mycorrhiza of *L. bicolor–P. resinosa*. By using a mycorrhizal cDNA library, we were able to identify cDNAs coding for proteins interacting with the fungal Ras gene product in the established mycorrhiza. We named this line of clones *Rythm* clones (Ras-interacting yeast two-hybrid mycorrhizal clones).

RythmA has an open reading frame coding for AP180-like proteins reported in other systems. Although *AP180*-like

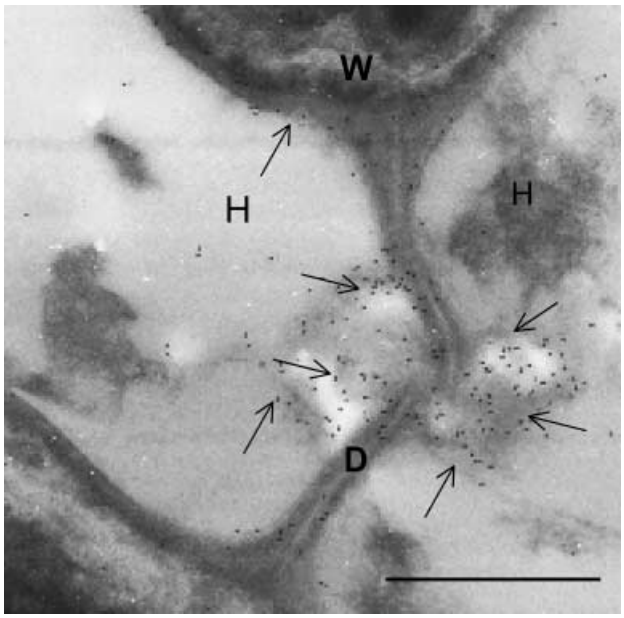

Fig. 5 Immunolocalization of Lbras protein (bait protein in yeast two-hybrid assays) in the mycorrhizal tissue (4 months old) through transmission electron microscopy analysis. Antibody generated against the recombinant Lbras was used in the analysis (Sundaram *et al*., 2001). H and W refer to the fungal hyphal cytoplasmic region and root wall while D refers to fungal dolipore-septum, respectively. Bars correspond to 400 nm. Arrows indicate signals corresponding to Lbras. The labeling of LbRAS at dolipore septa was observed frequently (60% of the sections) in the Hartig-net region, especially in fungal cells in close contact with the inner cortical cells.

genes have been cloned previously from mammalian systems and *S. cerevisiae*, this is the first report of identification of *Ap180*-like gene from a mycorrhizal fungus. The AP180 protein has been shown to play roles in assembly of clathrin-coated vesicles through protein–protein interactions (Hao *et al*., 1999). It is also implicated in cargo sorting in coated vesicles through its interaction with GTPases (De Camilli *et al*., 1996). The mycorrhizal interactions have been considered to involve considerable exchange of nutrients, signals and ligands between the partners. Turnover of vesicles during the ectomycorrhizal phenomenon and regulation of vesicular traffic proteins during *L. bicolor–P. resinosa* ectomycorrhizal symbiotic interactions have also been previously reported (Kim *et al*., 1999b). Hence, the finding that a vesicular assembly/cargo traffic-associated protein is regulated during these interactions is consistent with previously reported studies. Lbras–RythmA two-hybrid interactions were further verified by transforming the interaction host strain of *S. cerevisiae* with pLbras and pRythmA plasmids simultaneously and checking the transformants for reporter gene expression. Southern analysis confirmed the fungal origin of *RythmA* and presence of possibly one or two copies of *RythmA* in the *L. bicolor* genome. This was further supported by the absence of any detectable *RythmA* expression in nonmycorrhizal red pine roots (Fig. 4b). *AP180* genes have been shown to be present as multiple variants in other eukaryotic systems (Nonet *et al*., 1999).

Northern analysis suggested that diffusible signals from host are required for the induction of *RythmA*. The fact that the *RythmA* clone came from a mycorrhizal library and the absence of any signal in Northern blots with RNA from free-living fungal mycelial tissue clearly demonstrate the differential expression of *RythmA* between the mycorrhizal and free-living fungal forms, as well as host-interacted, preinfection stage fungal mycelial tissue. Reverse transcription-PCR analysis (Fig. 3 Panel B) confirmed RythmA expression in mycorrhizal tissue and clearly indicate that *RythmA* expression is not an artifact of carbon starvation, which is bound to happen in the interaction in the absence of the host. Also, the Northern analysis indicates that even though there appears to be two copies of *RythmA* in *L. bicolor* genome, only one of them is expressed at detectable levels during the early stages of *L. bicolor–P. resinosa* preinfectious interactions. Thus, it is likely either the *RythmA* is one copy gene or the other copy may be differentially regulated. Our previous studies with the *L. bicolor–P. resinosa* system has shown induction of fungal genes as early as 6 h after interaction (Kim *et al*., 1998, 1999a). Induction of *RythmA* after 36 h of interaction with the host, as seen from the Northern analysis, suggests that *RythmA* is not one of the early genes turned ON during such interactions. It is possible that turning ON of some other gene(s) during the preinfection stage interactions could lead to regulation of *RythmA* expression. It is consistent with the fact that Lbras with which RythmA exhibits yeast two-hybrid interactions also shows differential expression within 24–48 h of *L. bicolor's* symbiotic interaction with red pine, with a peak expression at 36 h (Fig. 3c) (Sundaram *et al*., 2001). More recently, we were able to detect expression of *LbRAS* earlier than 6 h into interaction then reappear after 24 h (data not shown). It appears that *RythmA* expression closely follows *LbRAS* expression after 24 h.

Phylogenetic analysis with PAUP yielded an unrooted tree (Fig. 4) that clustered together AP180-like proteins from *C. elegans*, *A. thaliana* and *S. cerevisiae* while grouping RythmA and Ap180-like proteins from *Rattus norvegicus*, *Xenopus laevis*, *Drosophila melanogaster* and *Loligo paelei* into a different cluster. BLAST analysis showed RythmA to have highest sequence homology with its *A. thaliana* counterpart. This is not really surprising owing to the fact that BLAST analysis is based on overall similarities in the sequences while parsimony analysis looks at amino acids that are different among homologous sequences (Farris *et al*., 1983; Berlocher & Swofford, 1997). In essence, the phylogenetic analysis using parsimony indicates the potential evolutionary path of closely related sequences and, in this case, it shows RythmA and AP180-like proteins from *R. norvegicus*, *X. laevis*, *D. melanogaster* and *L. paelei* to have evolved differently from their *A. thaliana*, *S. cerevisiae* and *C. elegans* counterparts. This is consistent with previously reported phylogenetic analyses on other

symbiosis-regulated genes from *L. bicolor* (Sundaram *et al.*, 2001). Searches done on genome databases of *F. graminearum*, *A. fumigatus* and *Neurospora crassa* yielded no significant matches. It is likely that while these fungi may have proteins that have similar functions, their sequence homology may be divergent. Thus, it is not yet possible to determine the evolutionary relatedness of these genes in fungi.

Although there is documented evidence of AP180–GTPase interactions, the nature or roles of Lbras–RythmA interactions during ectomycorrhizal symbiosis is not clear. Transport of Ras and other GTPases in clathrin-coated vesicles or endocytic vesicles to facilitate rapid long range signaling has been previously reported (Lenhard *et al.*, 1991; Kholodenko, 2003). It is intriguing to note that such a transport has been reported in human placenta, which in a way can be considered as a nutrient exchange organ (Lenhard *et al.*, 1991).

Our previous work (Sundaram *et al.*, 2001) with Lbras antibodies had shown the localization of the bait protein (Lbras) in established *L. bicolor–P. resinosa* ectomycorrhiza tissue. In addition to signals along the cell membranes (Sundaram *et al.*, 2001), we were also able to detect clustering of signals near fungal dolipore-septum region in the Hartig net area, indicating the presence of Lbras (protein used as bait in the yeast two-hybrid interactions) in this highly dynamic region inside the fungal hyphae (Fig. 5). These observations point to the accumulation of Lbras near the fungal dolipore-septum in established ectomycorrhizal tissue, suggesting intercellular movement of Lbras inside the fungal hyphae. There have been reports of intercellular movement of materials inside mycorrhizal fungus towards the hyphal tip (Cole *et al.*, 1998). In the light of the above, it is tempting to suggest that RythmA–Lbras interactions might result in intercellular transport of Lbras inside the mycorrhizal organ. There have been previous reports of exchange of materials between adjacent cells via septal pores in the mycorrhizal fungus *Pisolithus tinctorius* (Shepherd *et al.*, 1993). The fact that such intercellular transports have been suggested to be directional (Shepherd *et al.*, 1993) indicate that Lbras movement in ectomycorrhiza could be directional. Lbras has been suggested to perform different functions at different stages of mycorrhizal interactions between *L. bicolor* and *P. resinosa* (Sundaram *et al.*, 2001). Cloning of *RythmA* from a cDNA library from a functional ectomycorrhizal tissue coupled with the detection of signals in the Northern blot analysis of mycelial tissues from preinfection stage of mycorrhizal interactions might suggests that Lbras performs different tasks at different stages of mycorrhizal development and functioning. While Lbras–RythmA interactions could lead to the transport of Lbras and Lbras mediated cargo sorting into clathrin-coated vesicles in established mycorrhizal tissues, other Lbras-mediated cellular signaling cascades could occur during early stages of mycorrhizal formation. Ras being a signaling protein, it is entirely possible that transportation of Ras proteins towards hyphal tip during preinfection interactions might lead to increased perception of host signals leading to an appropriate cascading response, eventually resulting in the growth of hyphal tip towards the host root. Performing yeast two-hybrid interactions with tissues from early stages of *L. bicolor–P. resinosa* could lead to isolation of genes coding for proteins interacting with Ras proteins in cellular signaling cascades as described (Vojtek & Channing, 1998). These efforts are currently underway.

It is also possible that Lbras–RythmA interactions might assist vesicular assembly and cargo sorting in general. GTPases such as Scaffold associated region (SAR) and ARF belonging to the Ras family have been shown to be involved along with AP180 in the assembly of clathrin-coated vesicles (De Camilli *et al.*, 1996). There have been reports of myristylated GTPases regulating vesicular assembly and lateral segregation (De Camilli *et al.*, 1996; Novick & Guo, 2002). Motif search with PROSITE showed the presence of myristylation site in Lbras as well as a region found conserved among ARF family of proteins (Fig. 2b). It is not clear whether Lbras–RythmA interactions lead to a vesicular transport of Lbras or whether Lbras mediates vesicular assembly in *L. bicolor*, thus assuming the roles of other GTPases belonging to the Ras family such as SAR and ARF. As all the reports on AP180 interactions so far have come from either mammalian systems and *S. cerevisiae* and none from filamentous fungi, it is difficult to speculate the specific roles of RythmA interactions in ectomycorrhizal symbiosis. It is plausible that RythmA has different roles during early ectomycorrhizal interactions and is involved in a different set of roles in established mycorrhiza. Further analyses such as immunoprecipitation and localization experiments are needed to determine the roles of RythmA in ectomycorrhizal symbiosis. We are currently preparing RythmA protein in *E. coli*, to use it to produce antibodies. These antibodies will provide a better tool to understand the role of RythmA as well as RythmA–Lbras interactions in ectomycorrhizal symbiosis using protein pull down experiments.

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